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Resistance of *Spiroplasma citri* Lines to the Virus SVTS2 Is Associated with Integration of Viral DNA Sequences into Host Chromosomal and Extrachromosomal DNA

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Spiroplasmavirus SVTS2, isolated from *Spiroplasma melliferum* TS2, produces plaques when inoculated onto lawns of *Spiroplasma citri* M200H, a derivative of the type strain Maroc R8A2. *S. citri* strains MR2 and MR3, originally selected as colonies growing within plaques on a lawn of M200H inoculated with SVTS2, were resistant to SVTS2. Genomic DNA fingerprints and electrophoretic protein profiles of M200H, MR2, and MR3 were similar, but three proteins present in M200H were missing or significantly reduced in both resistant lines. None of these three polypeptides reacted with antiserum against *S. citri* membrane proteins, indicating that they probably are not surface-located virus receptors. Electroporation with SVTS2 DNA produced 1.5×10^5 transfectants per μg of DNA in M200H but none in MR2 or MR3, suggesting that resistance may result from inhibition of viral replication. The digestion patterns of the extrachromosomal double-stranded (ds) DNA of these lines were similar. Three *Taq*I fragments of MR2 extrachromosomal DNA that were not present in M200H extrachromosomal DNA hybridized strongly to an SVTS2 probe, and two of these fragments plus an additional one hybridized with the MR3 extrachromosomal DNA, indicating that a fragment of SVTS2 DNA was present in the extrachromosomal ds DNA of MR2 and MR3 but not of M200H. When the restricted genomes of all three lines were probed with SVTS2 DNA, strong hybridization to two *Eco*RI fragments of chromosomal MR2 and MR3 DNA but not M200H DNA indicated that SVTS2 DNA had integrated into the genomes of MR2 and MR3 but not of M200H. When MR3 extrachromosomal ds DNA containing a 2.1-kb SVTS2 DNA fragment was transfected into M200H, the transformed spiroplasmas were resistant to SVTS2. These results suggest that SVTS2 DNA fragments, possibly integrated into the chromosomal or extrachromosomal DNA of a previously susceptible spiroplasma, may function as viral incompatibility elements, providing resistance to superinfection by SVTS2.

Spiroplasmas, wall-less prokaryotes placed taxonomically with mycoplasmas in the class *Mollicutes*, contain circular genomes. Extrachromosomal DNA may be present in the form of plasmids, viruses, or both. Four groups of spiroplasma viruses (SpV1 through SpV4) have been described (2). The complete nucleotide sequences of SpV1-R8A2B from *Spiroplasma citri* R8A2 and SpV4 from *Spiroplasma melliferum* have been determined (29). The replicative form (RF) of SpV1-R8A2B has been used as a cloning vector for gene expression in spiroplasmas (17, 37).

Some spiroplasma strains are resistant to infection by specific viruses. Two mechanisms accounting for these resistances have been proposed. Bove et al. (2) hypothesized that, for two spontaneous mutants of *S. melliferum* which were resistant to the virus SpV4 but susceptible to its transfected DNA, resistance was due to failure of the virion to adsorb to or to penetrate the spiroplasma. Alteration of surface receptors as a mechanism of resistance also is consistent with the dramatic changes in surface properties upon SpV3-type viral infection of sex ratio-altering spiroplasmas of *Drosophila* species (23). A second proposed mechanism for virus resistance is that viral replication is compromised by the integration of copies of the viral genome into the spiroplasma chromosome. Integration of a complete copy of the SpV3-ai genome into *S. citri* SPA to establish a lysogen resulted in immunity to infection by SpV3-ai but not by related SpV3-type viruses (7). Immunity in this case was also due in part to a surface alteration that resulted in failure of virions to adsorb to the spiroplasma. A combination of replication interference and surface alterations may thus condition some resistances. However, although the genome of *S. citri* R8A2 contains multiple dispersed sequences closely resembling entire SpV1-78 viral DNA genomes (15, 27), R8A2 is susceptible to SpV1-78 infection, perhaps indicating that the integrated genomes differ slightly but significantly from that of SpV1-78 (2).

In the studies described in the preceding paragraph, the resistant spiroplasma strains had been derived from strains that produced the virus being investigated or a closely related virus (2, 6). As a result, the genomes of these resistant spiroplasma strains already contained complete (2) or partial (6) copies of the viral genomes, a fact that complicates the interpretation of results.

Virions of SVTS2, an SpV1-type virus isolated from the honeybee-inhabiting spiroplasma *S. melliferum* TS2 (19), are rod-shaped particles containing a 6.5-kb circular, single-stranded DNA. Although most other spiroplasma viruses characterized to date infect spiroplasmas of only one species, SVTS2 also forms plaques when inoculated onto lawns of the phytopathogen *S. citri* M200H (18). Two lines of *S. citri*, designated MR2 and MR3, apparently resistant to SVTS2 infection, were isolated from within plaques on lawns of strain M200H inoculated with SVTS2. These lines thus provide an

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opportunity to explore spiroplasma resistance to viruses in the absence of prior association of the spiroplasma with the virus to which it develops resistance. For the purposes of this discussion, we use the term resistance to indicate reduction or elimination of plaque formation in lawns of virus-inoculated spiroplasmas.

In the experiments described here, we characterized the relationship of the virus SVTS2 with the susceptible parent line M200H and with virus-resistant *S. citri* lines MR2 and MR3. Spiroplasma surface alterations and virus replication interference were investigated as possible mechanisms of spiroplasma resistance to the virus. The hypothesis that resistance is due to failure of the virus particles to adsorb to the spiroplasma surface due to loss of a virus receptor in the resistant spiroplasma lines was explored by using one- and two-dimensional gel electrophoresis of susceptible and resistant lines to identify profile differences which could reflect the loss of a virus-binding protein. We also showed that the resistant lines did not sustain viral replication even after transfection with purified virus DNA. That the virus resistance of lines MR2 and MR3 is related to the introduction of virus sequences into the host and possibly to its integration into host DNA was examined by searching for virus sequences in the genomic and extrachromosomal DNA of susceptible and resistant lines and by testing the effect on virus susceptibility of introduction of a virus DNA fragment into the susceptible line. The results showed that resistance was associated with integration of viral sequences in the host spiroplasma DNA.

(Preliminary reports of this work have been published [34, 36].)

MATERIALS AND METHODS

Source of spiroplasmas and viruses. *S. citri* Maroc R8A2 was isolated originally from citrus in Morocco (31); M200H was derived from strain R8A2 by 200 passages in subculture; MR2 and MR3, lines resistant to spiroplasma virus SVTS2, were derived from M200H (described below). *S. citri* BR3-3X, BR5-3X, and BR6-3X were isolated from Illinois horseradish with brittle root symptoms (10) and triply single-cell cloned. *S. melliferum* TS2 and AS576 were isolated from honeybees (3, 5), and *Spiroplasma floricola* 23-6 was isolated from a flower surface (4). All strains were cultured as described by Fletcher et al. (10) and stored in broth at −70°C. Virus SVTS2 was isolated from *S. melliferum* TS2 and amplified in *S. citri* M200H as described by McCammon and Davis (19, 20). Virus SVBR3 was isolated from *S. citri* BR3-3X (33, 35).

Isolation of virus-resistant lines and assay for virus resistance. A lawn of *S. citri* M200H was prepared by spreading 100 μl of broth culture (approximately 10^8 cells per ml) onto a 6-cm LD8 agar plate. The plate was incubated overnight at 31°C, then 50 μl of SVTS2 preparation (10^{12} PFU/ml) was distributed over the lawn, and the plate was incubated at 31°C for 2 days for development of plaques. Two colonies growing within the plaque boundaries, designated MR2 and MR3, were transferred to LD8 broth and frozen in aliquots at −70°C.

Virus isolation and purification. *S. melliferum* TS2 was grown to a titer of 5×10^8 to 1×10^9 cells per ml in 1 liter of LD8 broth. For virus isolation, cells were pelleted at 10,000 × *g* and 4°C for 30 min. The supernatant was brought to 0.2% Triton X-100 and incubated overnight at 4°C. This suspension was centrifuged at 95,000 × *g* and 5°C for 3 h, and the pellet was resuspended in 10 mM Tris-HCl (pH 7.9) overnight. For each milliliter of virus preparation, 0.387 g of CsCl was added, and the mixture was centrifuged at 105,000 × *g* and 20°C for 24 to 48 h. The virus band was removed from the tube and dialyzed against 10 mM Tris-HCl (pH 7.9) at 4°C for 48 to 73 h with three changes of buffer. Virus titer was determined by plaque assay.

Plaque assay. *S. citri* was grown in LD8 broth to a titer of approximately 2×10^8 cells per ml. One hundred microliters of culture was spread onto agar-solidified LD8 medium in 6-cm plates and allowed to dry, covered, at room temperature for 1 to 2 h. A volume of 50 μl of virus suspension at approximately 4×10^6 PFU/ml was distributed over the lawn. Plates were inverted and incubated at 31°C until plaques were visible (2 to 4 days).

Extraction of spiroplasma total cell proteins and membrane proteins. Log-phase cultures (approximately 10^{10} cells per ml) of strains M200H, MR2, and MR3 were prepared for extraction of cell proteins as described previously (10). The Triton X-114 phase separation method of Bordier (1), as modified for mycoplasmas by Riethman et al. (30) and Fletcher and Wijetunga (11), was used to separate spiroplasma hydrophobic membrane proteins from hydrophilic cytoplasmic proteins. The starting sample was 1 ml of an *S. citri* protein preparation at 1 mg/ml (Bio-Rad protein assay). Separation steps were repeated twice to ensure complete separation of the proteins in the two phases.

SDS-PAGE and Western immunoblotting. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cells were pelleted, solubilized, and electrophoresed as previously described (10) in 10% acrylamide slab gels (1.5 mm by 14 cm). Samples containing 10 μg of protein per well were electrophoresed for 15 h at 7 to 8 mA constant current. Bands were visualized with a silver stain (21), or proteins were transferred to nitrocellulose for Western blotting. For two-dimensional gels, proteins were subjected to isoelectric focusing (IEF) followed by SDS-PAGE (22, 33). The pelleted spiroplasma cell preparation, resuspended in 0.07 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5 (HEPES buffer) containing 10% sucrose, was centrifuged at 15,000 × *g*, and pellets were resuspended in IEF buffer (9 M urea, 4% [wt/vol] CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}, 0.5% [wt/vol] dithiothreitol, 5% [vol/vol] Biolyte 3/10, 2% [vol/vol] Biolyte 5/7 [Bio-Rad Laboratories, Richmond, Calif.]). Samples (25 μg of protein per tube) were loaded onto IEF tube gels (0.25 by 8.5 cm) and electrofocused at 400 V for 12 to 18 h and then at 800 V for 2 h. Gels were extruded from the tubes, frozen on dry ice, and stored at −20°C. For the second dimension, an IEF gel was equilibrated in SDS reducing buffer (0.5 M Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.75% dithiothreitol, 0.005% bromophenol blue) and overlaid onto an SDS–10% PAGE slab gel. The second-dimension gel was electrophoresed for 15 h at 7 to 8 mA constant current in a gel (16 cm long by 14 cm wide by 1.5 mm thick), and proteins were visualized with silver stain (21). After SDS-PAGE, the separated proteins were Western blotted, and the blots were developed with antiserum developed previously against *S. citri* BR3-3X membrane proteins (12).

Genomic DNA purification and fingerprinting. *S. citri* genomic DNA was isolated by the method of Williamson et al. (39) except that centrifugation of the CsCl gradients was done at 239,000 × *g* for 15 h. DNA recovered from the gradient was subjected to two cycles of phenol extraction and ethanol precipitation. The fingerprinting method of Herring et al. (13) was used. Genomic DNA was digested with *Eco*RI and electrophoresed by SDS-PAGE on slab gels (1.5 mm by 14 cm) of 7% polyacrylamide for 18 to 20 h at 22 to 25 mA constant current in electrode buffer (0.036 M Tris, 0.03 M sodium dihydrogen phosphate, 0.001 M EDTA [pH 7.8]).

Purification of spiroplasma virus DNA. A modification of the method of McCammon and Davis (20) was used to purify viral DNA. The pellet from 1.0 liter of a log-phase culture of spiroplasmas was resuspended in 4.0 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Four grams of CsCl and 0.4 ml of ethidium bromide (10 mg/ml) were added. The density of the solution was adjusted to 1.55 g/ml, and it was centrifuged at 239,000 × *g* in a Beckman VTi type 65 rotor for 15 h. The DNA was recovered by ethanol precipitation and dried. The final pellet, resuspended in 50 μl of TE buffer, was stored at −20°C.

Restriction mapping of SVTS2 DNA. Restriction enzymes were used following the recommendations of the manufacturer (GIBCO BRL). For double digestions, reaction mixtures were incubated at 37°C for 1 h except for those with *Bst*yI and *Taq*I, which were incubated at 60 or 65°C. The digested DNA was precipitated with ethanol before being digested with a second enzyme. The digested DNA was analyzed by electrophoresis in 0.7% agarose gels with TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]).

Purification of spiroplasma extrachromosomal ds DNA, including virus RF. To maximize virus yield, 5 ml of M200H culture (log phase, approximately 10^8 cells per ml) was incubated with SVTS2 (10^{14} PFU/ml) at a multiplicity of infection (MOI) of 100 for 1 to 2 days and then transferred to 100 ml of cultured M200H. After similar sequential transfers to 0.5- and 1.0-liter M200H cultures, the spiroplasma cells were pelleted at 13,000 × *g* for 35 min at 4°C. Supercoiled DNA was recovered from the cells by alkaline-SDS lysis (32) and further purified by CsCl gradient centrifugation as described above. Recovered DNA was dissolved, extracted with phenol, and precipitated with ethanol. Indigenous extrachromosomal double-stranded (ds) DNA from M200H, MR2, MR3, and BR3 was obtained by the same purification protocol.

Transfection of *S. citri* with viral DNA. Transfection with polyethylene glycol (PEG) was done as previously described (20). For electroporation, the procedure of Stamburski et al. (37) was slightly modified. *S. citri* cells (10 ml at 10^9 cells per ml) were collected by centrifugation and resuspended in 0.8 ml of 8 mM HEPES buffer. The cell suspension was mixed with 10^4 to 10^6 pg of SVTS2 DNA, placed into a chilled 0.4-cm cuvette, and incubated on ice for 10 min. The Gene Pulser (Bio-Rad) conditions were 3 μF capacitance, 2.5 kV set volts, 1,000 Ω, and 1-ms pulses. Two pulses were applied. After a 10-min incubation at room temperature, the cells (100 μl per plate) were dropped onto a previously prepared lawn of an *S. citri* test strain. Plates were checked for plaques after 1 to 2 days.

Molecular cloning and hybridization of SVTS2 and SVBR3 RF DNA. Standard methods were used in working with RF DNA (32). SVTS2 and SVBR3 RF DNAs partially digested with *Sau*3AI were ligated with *Bam*HI-digested and calf intestinal phosphatase-treated Bluescript KS(+), and the ligation mixture was used to transform *Escherichia coli* (Library Efficiency DH5α competent cells; Life Technologies Inc.) by heat shock (42°C, 2 min). Recombinant plasmids were isolated by alkaline-SDS extraction and purified by ethidium bromide-CsCl gradient centrifugation, phenol extraction, and ethanol precipitation. Purified DNA was labeled with ^{32}P by nick translation, and standard methods of Southern blotting and hybridization were used (32). Prehybridization was done at 65°C for 2 h. Hybridization was done overnight at 65°C, followed by two washes (room

TABLE 1. Plaque assay for infectivity of SVTS2 on *S. citri* M200H, MR2, and MR3[a]

Lawn	SVTS2 (PFU/ml)		Tris buffer	None[b]
	4.2×10^4	4.2×10^6		
M200H	+	+	−	−
MR2	−	−	−	−
MR3	−	−	−	−

[a] These data were collected from three different experiments. Each combination was tested on a total of 26 plates. Symbols: +, presence of clear plaques in lawns; −, no plaque formation.
[b] No virus or buffer was added.

temperature for 30 min each) in 2× SSC (32)–0.1% SDS and then two washes (65°C for 15 min each) in 0.1× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

RESULTS

Resistance of *S. citri* lines MR2 and MR3 to SVTS2 infection and genomic DNA fingerprinting of spiroplasma lines. *S. citri* MR2 and MR3 were originally isolated from colonies growing within plaque boundaries on a lawn of M200H inoculated with virus SVTS2. After inoculation with SVTS2, lawns of *S. citri* M200H formed cleared plaques, but MR2 and MR3 lawns did not, even when the virus concentration was increased to 4.2×10^6 PFU/ml, 100-fold higher than that used for M200H infection (Table 1). This experiment was repeated three times. To assure ourselves that MR2 and MR3 arose from M200H, the *Eco*RI restriction fingerprint patterns of the genomic DNA of the three lines were compared and found to be identical (Fig. 1). Since *S. citri* lines derived by separate multiple passages from the same original strain (e.g., BR3-3X) accumulated detectable fingerprint differences (33), the identity of the patterns is consistent with the origin of MR2 and MR3 from M200H.

Restriction map of SVTS2 DNA. Restriction enzymes *Alu*I, *Bst*yI, *Dde*I, *Dra*I, *Eco*RI, *Hin*cIII, *Hin*fI, *Msp*I, and *Sau*3AI digested the SVTS2 DNA, while *Bam*HI, *Bgl*II, *Bst*xI, *Cla*I, *Hin*dIII, *Hha*I, *Pvu*II, *Sac*I, and *Sma*I did not. A restriction map of the virus was constructed by double digestions with restriction enzymes *Sau*3AI, *Hin*fI, *Taq*I, *Hin*cIII, *Hpa*II, and *Eco*RI (Fig. 2).

Identification of protein differences among M200H, MR2, and MR3. To investigate the hypothesis of alteration of surface receptors as a resistance mechanism, we evaluated the protein profiles of susceptible and resistant *S. citri* lines. In one-dimensional SDS-PAGE, 46 bands were distinguished in lanes loaded with total proteins of *S. citri* M200H. No significant differences were seen between the M200H profile and those of MR2 or MR3 (Fig. 3A). The two-dimensional IEF/SDS-PAGE pattern of *S. citri* M200H consisted of 155 distinguishable protein spots, while MR2 had 153 and MR3 had 154 (Fig. 3B, C, and D). Three protein spots present in M200H were missing or significantly reduced in MR2 and MR3. The amount of spot P1 (42.7 kDa) was significantly reduced in MR3 and the spot was missing in MR2, spot P2 (also 42.7 kDa) was missing in both MR2 and MR3, and the amount of spot P3 (36.5 kDa) was significantly reduced in both MR2 and MR3. These differences were reproducibly observed in more than 20 replications of the experiment. Western blots of one-dimensional gels, developed with antiserum against *S. citri* BR3-3X membrane proteins, showed no differences among M200H, MR2 (Fig. 4A), and MR3 (data not shown), although an 89-kDa membrane protein of *S. citri* BR3-3X was missing in M200H, MR2, and MR3.

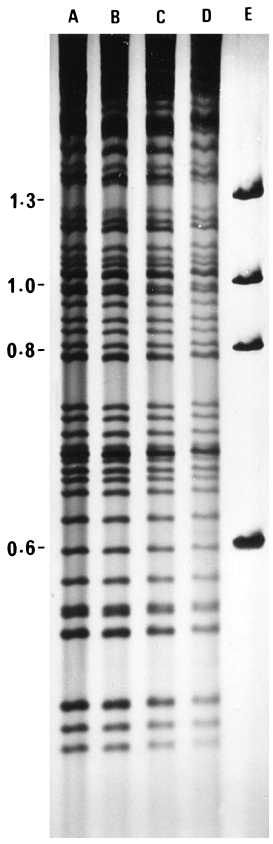

FIG. 1. Genomic DNA fingerprinting of *S. citri* lines by *Eco*RI digestion. Lanes: A, MR3; B, MR2; C, M200H; D, R8A2; E, standard molecular markers. Sizes are shown in kilobases.

Blots of two-dimensional gels of *S. citri* M200H probed with antiserum against *S. citri* BR3-3X membrane proteins contained spots designated P4 to P10 (Fig. 4B) in all three strains. Spots P1, P2, and P3 were not detected with this antiserum. P7 was identified as spiralin in blots probed with antiserum against the spiralin of *S. citri* BR3 (data not shown).

Absence of transfection of mutant *S. citri* lines with SVTS2 DNA. If the loss of a virus receptor protein were the mechanism of resistance in the mutant *S. citri* lines, then viral DNA

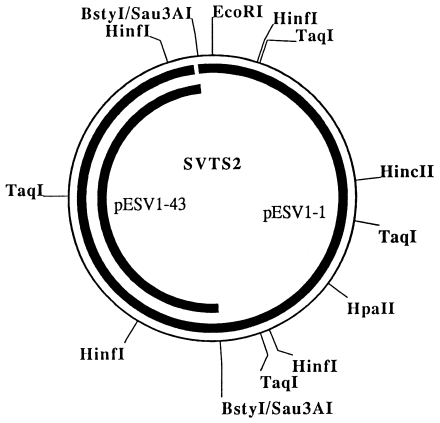

FIG. 2. Restriction map of SVTS2 genomic DNA. Solid bars indicate sections cloned in plasmids pESV1-43 and pESV1-1. Total size, 6,500 bp.

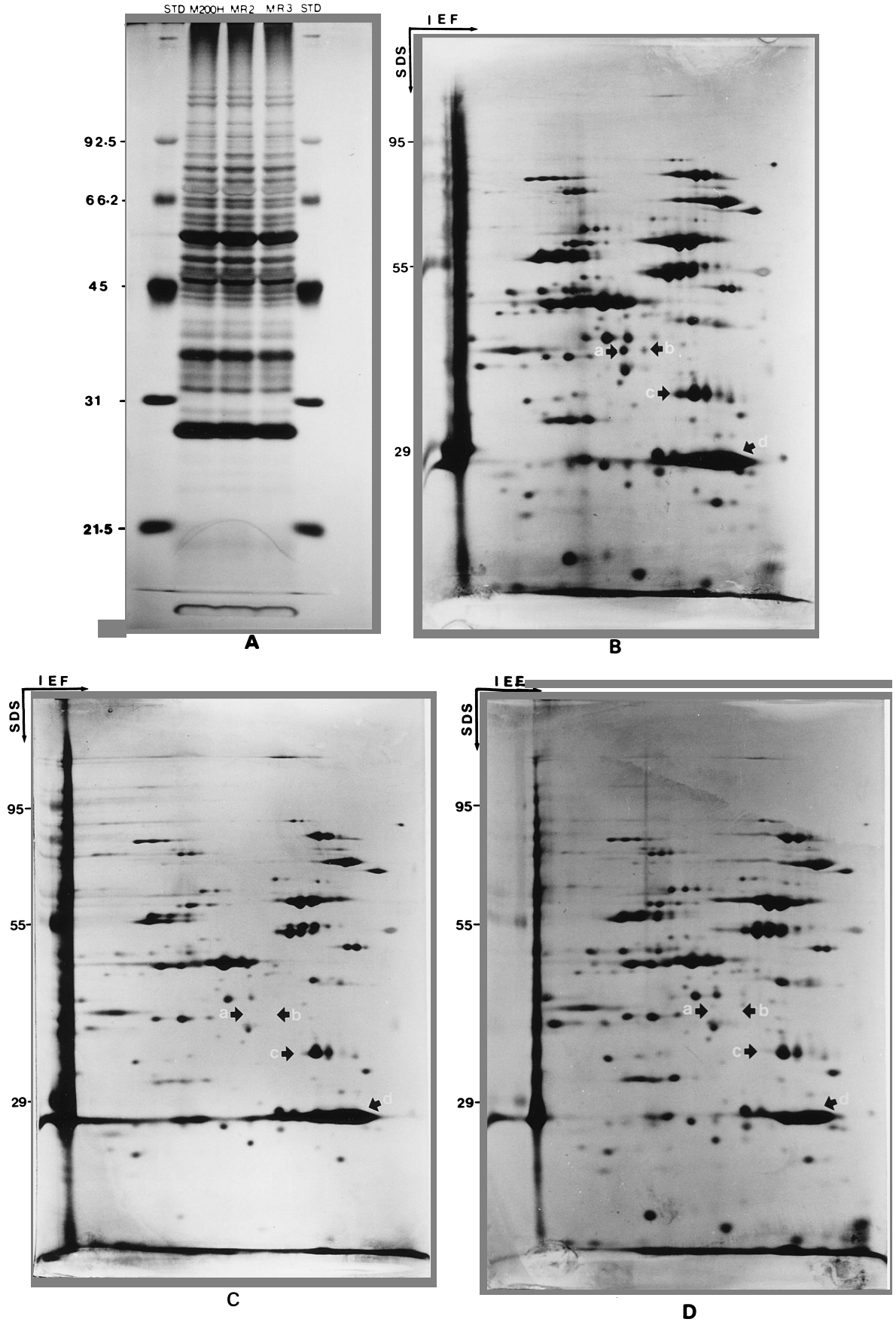

FIG. 3. Electrophoresis of total proteins of *S. citri* M200H and two virus-resistant lines, MR2 and MR3. (A) One-dimensional SDS-PAGE. Lanes STD, standard molecular size markers (in kilodaltons). (B, C, and D) Two-dimensional IEF/SDS-PAGE of M200H (B), MR2 (C), and MR3 (D). Arrows a to c point to spots P1, P2, and P3, respectively, which are not equally represented in all three lines; arrow d points to spiralin.

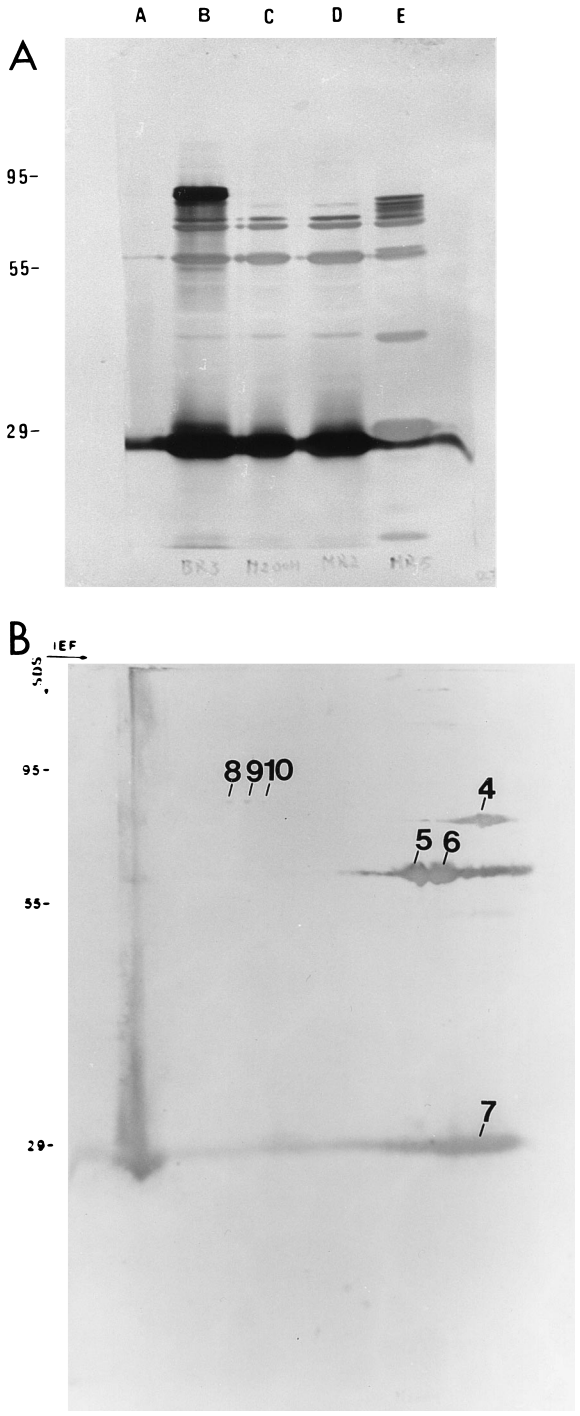

FIG. 4. Western blot of one-dimensional SDS-PAGE and two-dimensional IEF/SDS-PAGE of *S. citri* lines probed with antiserum specific for *S. citri* BR3-3X membrane proteins. (A) SDS-PAGE. Lanes: A, size standards (in kilodaltons); B, *S. citri* BR3; C, *S. citri* M200H; D, *S. citri* MR2; E, *S. melliferum* MR5. (B) IEF/SDS-PAGE of M200H. Spots P4 to P10 are marked.

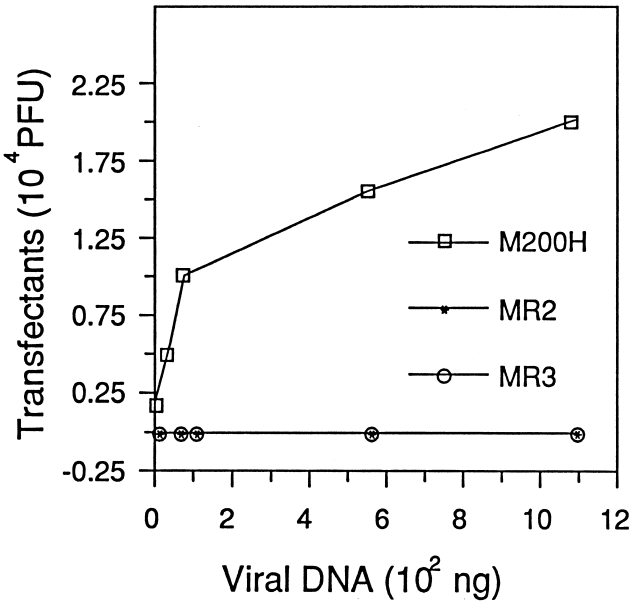

FIG. 5. Transfection of *S. citri* M200H, MR2, and MR3 with SVTS2 DNA.

should replicate normally following its introduction by transfection into those lines. We used both PEG and electroporation to transfect host spiroplasma cells with SVTS2 DNA. With 50% PEG, 194 plaques were observed in lawns of M200H inoculated with 1.4 μg of viral DNA, representing a transfection efficiency of 1.4×10^2 PFU/μg of viral DNA. No transfection was obtained under the same conditions in virus-resistant strains MR2 and MR3.

With electroporation, 1.6×10^3 and 1.6×10^4 plaques were produced in lawns of M200H transfected with 1.1×10^{-2} and 0.55 μg of SVTS2 DNA, respectively, representing transfection efficiencies of 1.5×10^5 and 2.9×10^4 transfectants per μg of DNA, respectively. The number of plaques obtained increased proportionately with increasing amounts of SVTS2 DNA (Fig. 5). No plaques were obtained with MR2 and MR3 in six replications of the experiment, although up to 1.1 μg of SVTS2 DNA was added; thus, if transfection occurred, the efficiency was <1 transfectant per μg of DNA.

Extrachromosomal differences among *S. citri* lines M200H, MR2, and MR3. To address the question of whether resistance to virus infection involves interference with virus replication, we investigated the extrachromosomal DNA of the susceptible and resistant lines. Filtrates of cell cultures of MR2, MR3, and M200H all produced plaques on lawns of the indicator strain *S. citri* M200H and on lawns of MR3, indicating that MR2, MR3, and M200H each contain a virus(es). We designated these native virus populations SVMR2, SVMR3, and SVM200H, respectively. Restriction fragments of extrachromosomal DNAs containing the RFs of SVTS2, SVM200H, SVMR2, and SVMR3 were compared. Digestion with *Dde*I produced patterns with nonstoichiometric distributions of fragments (Fig. 6). Several significant points can be deduced from the banding patterns; these are summarized in Table 2. Extrachromosomal DNA containing SVTS2 RF differed substantially from the DNAs from M200H and the derived lines, as evidenced by several TS2-specific and MR2-MR3-M200H-specific fragments (see Table 2, footnote *a*, codes a and c). However, the patterns of bands also suggested a relationship between the extrachromosomal DNAs of TS2 and of the M200H-derived lines (code d). As expected from their derivation from M200H, resistant lines MR2 and MR3 contained extrachromosomal DNAs related to those of M200H (code c). However, the resistant lines were still distinguishable from the parental line (code b). The patterns of restriction fragments further revealed that the two resistant lines differed in their extrachromosomal DNAs (code e). The patterns resulting from diges-

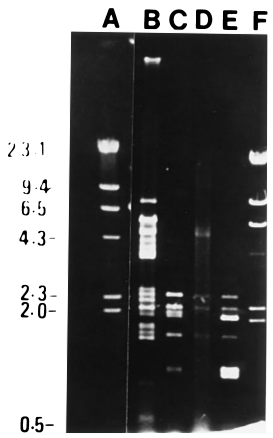

FIG. 6. *Dde*I digestion patterns of extrachromosomal DNA of spiroplasma strains carrying native virus SVTS2 (lane B), SVMR2 (lane D), SVMR3 (lane E), or SVM200H (lane C). Lanes A and F are standard molecular size markers (*Hin*dIII digest of lambda DNA) (in kilobases).

tion of the same extrachromosomal DNAs with *Sau*3AI, *Hin*dIII, *Taq*I, and *Alu*I were similar (data not shown).

SVTS2 DNA in the extrachromosomal ds DNA of MR2 and MR3. To investigate whether SVTS2 DNA is present in the extrachromosomal DNA of MR2 and MR3, we constructed an SVTS2 DNA probe. After cloning SVTS2 RF in *E. coli* with the vector Bluescript KS^+, clones with two types of plasmids were recovered. One type, designated pESV1-1, contained an insert of 6.5 kb, and the other, designated pESV1-43, contained an insert of 3.2 kb (Fig. 2 and 7). The 6.5-kb DNA fragment, labeled with ^{32}P for use as a DNA probe, hybridized to all *Alu*I-, *Taq*I-, and *Dde*I-digested fragments of SVTS2 DNA (data not shown), indicating that pESV1-1 represents a clone of the complete SVTS2 genome. That all of the SVTS2 RF restriction fragments reacted, even though their total length exceeded 6.5 kb in some cases (Fig. 6 and 8), indicates that the SVTS2 preparation is heterogeneous in its susceptibility to restriction. The heterogeneity could be due to different degrees of modification (33) or to variations in sequence.

S. citri extrachromosomal ds DNA (including native virus RF) was digested with *Taq*I, and a Southern blot was probed with clone pESV1-1 (Fig. 8). All of the ds DNA preparations, which are referred to here as SVM200H, SVMR2 and SVMR3, contained sequences hybridizing with SVTS2. Three weakly hybridizing bands, a 7.0-kb band in SVM200H only and two bands (approximately 2.3 and 3.0 kb) in SVM200H, SVMR2, and SVMR3, probably represent native virus DNA, since M200H was not previously exposed to SVTS2. Two bands

TABLE 2. Distribution of selected bands resulting from *Dde*I restriction digestion of extrachromosomal DNA (containing native virus RF) of *S. melliferum* TS2 or *S. citri* strains susceptible or resistant to SVTS2

*Dde*I fragment size (kb)	Fragment present in strain:				Code[a]
	TS2	M200H	MR2	MR3	
1.2	−	−	−	+	e
1.3	+	+	−	+	e
1.8	−	+	+	+	c
2.1	+	+	−	+	e
2.2	−	+	−	−	d
2.7	−	+	+	+	c
2.8	−	+	+	+	c
5.5	+	−	+	−	b, e
(10)[b]	+	−	−	−	a

[a] Codes: a, TS2 contains unique bands not present in the other lines; b, MR2, MR3, or both share a band with TS2 but not M200H; c, M200H, MR2, and MR3 contain numerous common bands; d, MR2, MR3, and TS2 are alike but differ from M200H; e, MR2 differs from MR3.

[b] Ten bands of different sizes but having the same distribution among the four spiroplasma strains.

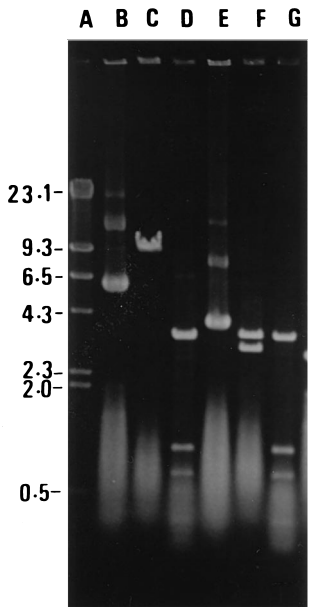

FIG. 7. Characterization of plasmid DNA clones of SVTS2 RF DNA. Plasmid DNAs from pESV-1 (lanes B, C, and D) and pESV-43 (lanes E, F, and G) were left undigested (lanes B and E) or digested with *Bam*HI (lanes C and F) or *Sau*3AI (lanes D and G). Lane A, standard molecular size markers (*Hin*dIII digest of lambda DNA) (in kilobases).

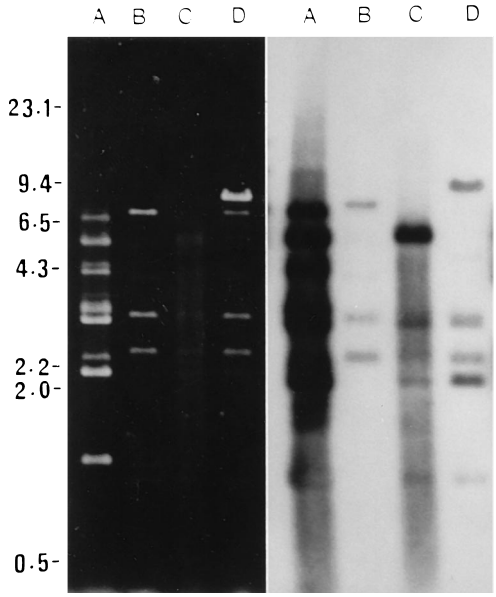

FIG. 8. Agarose gel (left panel) and Southern blot (right panel) of native viral RF DNA digested with *Taq*I and hybridized with the SVTS2 DNA probe. Lanes: A, SVTS2 RF DNA; B, SVM200H RF DNA; C, SVMR2 RF DNA; D, SVMR3 RF DNA. Sizes are shown in kilobases.

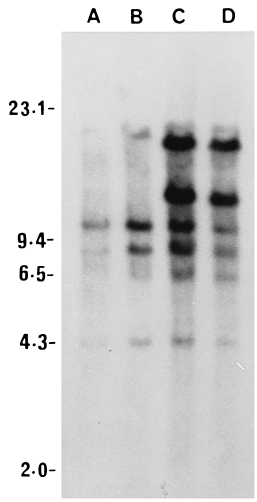

FIG. 9. Southern blot of *S. citri* chromosomal DNA digested with *Eco*RI and probed with SVTS2 DNA. Lanes: A, R8A2; B, M200H; C, MR2; D, MR3. Numbers at the left represent the positions of standard molecular size markers (*Hin*dIII digest of lambda DNA) (in kilobases).

corresponding in size to SVTS2 fragments (1.3 and 2.1 kb) hybridized in both SVMR2 and SVMR3 (Fig. 8, lanes C and D), although the same two bands were faint in the corresponding ethidium bromide-stained agarose gel; no hybridization to SVM200H was seen in this region of the gel (lane B). An additional band of hybridization was present in each of the resistant lines (7.6 kb in SVMR3 and 5.2 kb in SVMR2). The 5.2-kb SVMR2 band hybridized very strongly to the SVTS2 DNA probe, even though it was faint in the corresponding agarose gel. Three bands (1.3, 2.1, and 5.2 kb) not present in SVM200H corresponded to those obtained from digestion of SVTS2 RF DNA. Three replications of the experiment yielded similar results, which are consistent with the presence of fragments of SVTS2 DNA in extrachromosomal ds DNA molecules in MR2 and MR3.

SVTS2 DNA integration in the chromosomal DNA of MR2 and MR3. Chromosomal DNA of TS2, M200H, MR2, and MR3 was purified on a CsCl gradient and digested with *Eco*RI, and Southern blots were probed with SVTS2 DNA (Fig. 9). The absence of hybridization to a 7.5- to 8-kb band indicated that the chromosomal DNA was essentially free of extrachromosomal DNA contamination, since *Eco*RI digestion of SVM200H and SVMR2 DNA yielded single bands of that size. Five weak hybridization bands (4.3, 6.5, 8.4, 10.8, and 20.0 kb) in all of the strains (M200H, MR2, MR3, and R8A2) probably reflect chromosomal insertion of the respective strain's native virus DNA, since neither M200H nor R8A2 was previously exposed to SVTS2, but the native viruses all react weakly with SVTS2 probes (Fig. 8). Two bands (13.6 and 19.2 kb) hybridized very strongly to the probe in MR2 and MR3 but not in M200H, suggesting that part or all of the SVTS2 genomic DNA had inserted into the chromosome of MR2 and MR3 but not of M200H. The intensity of these bands suggests the presence of multiple copies of these viral DNA fragments in the chromosomal DNA. Since SVTS2 has only one *Eco*RI site, it is not clear whether the integrated DNA consists of entire viral genomes or only a virus-derived insertion sequence present in multiple copies in these *S. citri* genomes.

Susceptibility of MR2 and MR3 to the heterologous virus SVBR3. To test the hypothesis that the SVTS2 resistance of MR2 and MR3 results from the integration of SVTS2 sequences into the spiroplasma genome, it was necessary to demonstrate that MR2 and MR3 are capable of sustaining viral replication. Spiroplasma virus SVBR3, isolated from *S. citri* BR3-3X (33, 35), formed clear plaques 0.5 to 3.0 mm in diameter on lawns of M200H, MR2, and MR3 (Table 3). The morphology of the plaques was identical in these three lines. Southern blot hybridization with a cloned SVBR3 DNA probe (8.6 kb [the entire virus genome]) showed no hybridization to the chromosomal DNA of M200H, MR2, or MR3 (Fig. 10), indicating that the genomes of these lines had no SVBR3-related sequences.

TABLE 3. Analysis of natural virus infection, transfection with viral DNA, and viral DNA hybridization to spiroplasma chromosomal DNA

Species and strain	SVTS2			SVBR3		
	Natural infection[a]	Transfection[b]	DNA hybridization[c]	Natural infection	Transfection	DNA hybridization[d]
S. citri						
R8A2	+	ND	+	+	ND	−
M200H	+	+	+	+	+	−
MR2	−	−	+++	+	+	−
MR3	−	−	+++	+	+	−
BR3-3X	+	ND	+	−	ND	+++
BR5-3X	+	ND	+	−	ND	++
BR6-3X	+	ND	+	−	ND	++
S. melliferum						
TS2	−	ND	+++	ND	ND	±
AS576	−	ND	+++	−	−	±
S. floricola 23-6	+	ND	−	−	ND	−

[a] Natural infection refers to inoculation by adding virus suspension to host cell lawns. Symbols: +, production of plaques; −, no plaques; ND, not done.
[b] Transfection refers to inoculation by electroporation of viral DNA.
[c] With SVTS2 DNA probe. Symbols: +++, very strong hybridization; ++, strong hybridization; +, less strong hybridization; ±, weak hybridization; −, no hybridization.
[d] With SVBR3 DNA probe. Symbols: +++, very strong hybridization; ++, strong hybridization; +, less strong hybridization; ±, weak hybridization; −, no hybridization.

Modification of M200H to resistance by transformation with native virus SVMR3 RF DNA. If the hypothesis that the presence of viral DNA sequences is responsible for the resistance of lines MR2 and MR3 is correct, then introducing the same virus sequences into the susceptible parent, M200H, should confer resistance. A log-phase culture of M200H was transformed by electroporation with SVMR3 RF DNA containing a 2.1-kb SVTS2 fragment (Fig. 8). Isolated colonies growing within plaque boundaries in M200H lawns were transferred to and cultured in LD8 broth. One of these, designated M200H-SV1, was tested for susceptibility to SVTS2 infection. Plaques were formed on lawns of M200H but not M200H-SV1 following inoculation with SVTS2 at 5.6×10^8 PFU/ml (Table 4). With a higher concentration of SVTS2 (10^{14} PFU/ml), the lawn of M200H was almost cleared by confluent plaques, while a lawn of M200H-SV1 had only a few plaques.

Correlation of viral DNA presence in the spiroplasma genome and resistance to virus infection. DNA of SVTS2, which was originally isolated from *S. melliferum* TS2, strongly hybridized with the chromosomal DNA of *S. melliferum* TS2 and AS576, hybridized less strongly with the chromosomal DNA of *S. citri* (except that from strains MR2 and MR3), and did not hybridize with the chromosomal DNA of *S. floricola* 23-6 (Ta-

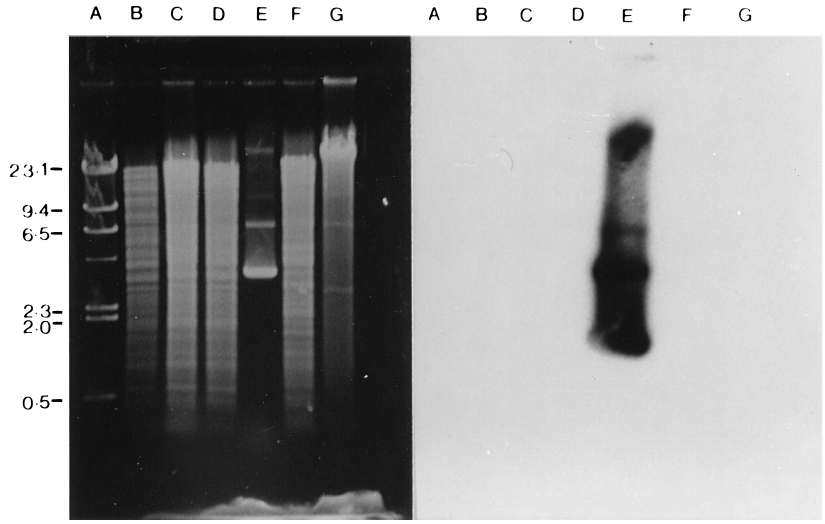

FIG. 10. Agarose gel (left panel) and Southern blot (right panel) of *S. citri* chromosomal DNA digested and probed with SVBR3 RF DNA. Lane A contains standard molecular size markers (*Hin*dIII digest of lambda DNA) (in kilobases). Lanes B to D were digested with *Eco*RI. Lanes: B, M200H; C, MR2; D, MR3; E, SVBR3; F and G, MR3 chromosomal DNA digested with *Hin*dIII and *Bam*HI, respectively.

ble 3). Host spiroplasma lawns of *S. citri* R8A2, M200H, BR3-3X, BR5-3X, and BR6-3X developed plaques after inoculation with SVTS2. However, lawns of *S. citri* MR2 and MR3 and *S. melliferum* TS2 and AS576 did not develop plaques.

The DNA of SVBR3 hybridized very strongly with the chromosomal DNA of *S. citri* BR3-3X, less strongly with the chromosomal DNA of *S. citri* BR5-3X and BR6-3X, weakly with the chromosomal DNA of *S. melliferum* TS2 and AS576, and not at all with the chromosomal DNA of *S. citri* lines R8A2, M200H, MR2, and MR3 or that of *S. floricola* 23-6. SVBR3 inoculation resulted in plaque formation on lawns of R8A2, M200H, MR2, and MR3. No plaques occurred in BR3-3X, BR5-3X, or BR6-3X.

In these experiments, there was a negative correlation between viral DNA hybridization to the spiroplasma chromosomal DNA and plaque production by that virus or a related virus. The only exceptions to this correlation were the failure of SVBR3 to infect *S. floricola* 23-6, to which there was no virus hybridization, and *S. melliferum* AS576, to which there was only weak hybridization. These cases probably reflect the origin of SVBR3 from a different spiroplasma species; at this time, SVTS2 is the only spiroplasma virus known to infect more than one spiroplasma species. Thus, virus inoculation resulted in the formation of plaques on lawns of spiroplasmas that did not contain corresponding viral DNA integrated into their genomes (and that hybridized weakly or not at all with the corresponding virus probe) but did not produce plaques when the host carried a complete or partial integrated viral DNA sequence (and reacted moderately or strongly with the virus probe).

TABLE 4. Susceptibility of *S. citri* M200H-SV1 to SVTS2

S. citri strain	Plaque formation[a]		
	SVTS2 (PFU/ml)		Tris buffer
	5.6×10^8	1×10^{14}	
M200H-SV1	−	+	−
M200H	++++	++++	−

[a] Plaque assay: −, no plaques produced; +, <5 plaques produced; ++++, >100 plaques produced.

DISCUSSION

Several mechanisms have been proposed to explain mollicute resistance to viruses. Dickinson and Townsend (7) suggested that the lysogenization of *S. citri* SpA-MD with SpV3-type virus ai results in a conversion of the cell surface, with loss of receptors for the virus. Bove et al. (2), reporting that *S. melliferum* BC-3, B63, G1R2, and G1R4 were resistant to infection by SpV4 virions but could be transfected by SpV4 DNA, suggested that resistance to infection by whole virus is due to lack of adsorption or penetration by the virions.

We used PAGE to seek protein differences among M200H, MR2, and MR3 which might reflect loss of a virus adhesin. Three polypeptides (P1, P2, and P3) were missing or significantly less concentrated in the *S. citri* lines resistant to SVTS2. The failure of these proteins to react with an antiserum prepared against *S. citri* membrane proteins suggests that polypeptides P1 to P3 are not membrane associated and therefore are not likely to be virus-binding proteins. However, the evidence is not conclusive, since strain BR3, which was used for rabbit immunization, may lack P1 to P3, or these BR3 polypeptides may have antigenic properties different from those of corresponding polypeptides in M200H. Also, since some membrane proteins do not fractionate into the detergent phase (24), the possibility that P1, P2, and P3 are membrane proteins absent from the detergent fraction used for immunization cannot be excluded.

M200H proteins P1, P2, and P3, if not virus receptors, may still be involved in virus resistance, perhaps as cofactors for virus binding. Alternatively, the changes in their prevalence could be a result, rather than a cause, of the switch to resistance. Oishi et al. (23) reported that the presence of virus affects the surface properties of the *Drosophila* sex ratio-altering spiroplasma.

However, factors other than prevention of virus binding may contribute to resistance to viruses. In experiments designed to bypass the virus adhesion step by electroporation of viral DNA into spiroplasma cells, SVTS2 DNA transfected *S. citri* M200H

at a high frequency but did not transfect lines MR2 and MR3. This result suggests that the absence of plaques reflects a phenomenon other than the loss of virus-binding spiroplasma surface proteins. One possible mechanism is a blockage of viral DNA replication.

In several cases, lysogeny of spiroplasma viruses has been correlated with viral integration into the host genome. DNA sequences of several spiroplasma viruses have been detected in the host chromosome (2, 7, 25–28, 40). For example, sequences of the SpV1 and SpV3 types of spiroplasma viruses are present in the chromosomal DNA of *S. citri*, *Spiroplasma kunkelii*, and *Spiroplasma phoeniceum* (2). Probably the entire SpV1 virus genome is present in the host genome (15). Dickinson and Townsend (7) found that the genome of *S. citri* spiroplasma SpA-MD (ai) contained a full complement of ai viral DNA and suggested that this insertion is the cause of lysogeny. *Acholeplasma laidlawii* cells lysogenized by mycoplasma virus L2 were found to have a single copy of the L2 genome integrated in the chromosome (9). Because our SVTS2-resistant *S. citri* lines MR2 and MR3 were derived from *S. citri* M200H, which had been inoculated with virus SVTS2, there was an opportunity for SVTS2 to leave part or all of the viral DNA in these cells. Our results are consistent with this explanation. Two *Eco*RI fragments (13.6 and 19.2 kb) that were present in the genomes of MR2 and MR3 but not M200H hybridized strongly to the viral DNA probe, indicating that part or all of the SVTS2 genome had integrated into the chromosomal DNA of MR2 and MR3 but not M200H. The fact that the introduction into M200H of a 2.1-kb fragment of SVTS2 DNA, carried as an insert in the DNA of SVMR3, resulted in virus resistance suggests that only part of the viral genome is required for resistance to result. If this is indeed the basis for the resistance of *S. citri* lines MR2 and MR3 to SVTS2, then the lack or reduction in level of three proteins (P1 to P3) in the virus-resistant lines MR2 and MR3 may be due to disruption of the genes encoding them by integration of viral DNA.

The message (if any) encoded by the SVTS2 DNA insertions is unknown. One possibility is that it encodes an integrase or is an insertion sequence element encoding a transposase. The mechanism of the viral DNA integration in MR2 and MR3 is unknown; however, others have found that spiroplasma virus SpV1-R8A2 B DNA has two open reading frames (ORFs) with varying sequence similarity to proteins catalyzing DNA insertion and excision events; ORF4 has limited similarity to the integrase of *Salmonella* phage P22 (26), and ORF3 has 25 to 30% identity to putative transposases of insertion elements IS*30*, IS*4351*, and IS*1086* (8). In addition, an inverted repeat present in SpV1-R8A2 B DNA is 76% identical to the terminal inverted repeat IS*1086*, an observation that led Dong et al. (8) to suggest that the ORF3 protein could act on the adjacent repeat to catalyze integration of the viral genome into the host chromosome. The possibility that SVTS2 insertions encode integrase or transposase is currently being investigated. Alternatively, enteric temperate phages, such as lambda, encode a repressor which confers immunity in their host cells to related phages, in addition to their function in maintaining lysogeny (16). Perhaps the integrated SVTS2 DNA fragments carried a similar gene for repression of viral DNA replication. When expressed in spiroplasmas, the repressor gene would inhibit replication of the same or a closely related virus. A third possibility for the role of SVTS2 DNA sequences in MR2 and MR3 is to function as small DNA "reduction sequences" that bind protein factors specifically necessary for SVTS2 replication. Such a mechanism occurs in the immunity of *E. coli* to ϕX174 bacteriophage infection; in this example, the reduction sequence can be located either in the chromosome or in extrachromosomal elements (38). That MR2 and MR3 each contain SVTS2 sequences in both of these locations is consistent with the latter scenario.

Though MR2 and MR3 are resistant to SVTS2, they are susceptible to a different virus, SVBR3 (from *S. citri*), which is serologically related to SVTS2 but does not hybridize with it (33, 36). This differential virus susceptibility is similar to the immunity of SpA-MD (ai) to SpV3-ai but not to the related viruses SpV3-ag and SpV3-AVa/3 (7). We found no hybridization to SVBR3 DNA in the genomes of MR2, MR3, or M200H. The SVTS2 DNA fragments present in the genomes of MR2 and MR3 apparently do not interfere with SVBR3 infection. Since there is no DNA sequence similarity between these two viruses at our level of detection, this finding further supports the hypothesis that viral DNA integration in the genome of *S. citri* is responsible for resistance to that virus but not to unrelated viruses. This observation also disproves the possibility that MR2 and MR3 are simply incompetent to replicate viral DNA.

Most temperate phages integrate into the genomes of their hosts, but some exist as extrachromosomal replication units or plasmids (14). The virus SVTS2 occurs in resistant spiroplasma lines as two forms, an extrachromosomal replication unit and an insertion in the host chromosome. To our knowledge, this is the first demonstration that part or all of an SpV1-type spiroplasma virus can exist simultaneously as integrated DNA and as an extrachromosomal replication unit. Our results support the hypothesis that spiroplasma virus SVTS2 fragments inserted into the *S. citri* chromosome and/or extrachromosomal DNA may function as viral incompatibility elements, providing resistance to superinfection by SVTS2.

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